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DEVELOPMENT OF SUCROSE-FERMENTING *ESCHERICHIA COLI* K-12 STRAINS FOR LOW-COST OVERPRODUCTION OF RECOMBINANT PROTEINS

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Escherichia coli K-12 strains were the most widely used microorganisms for carrying out genetic engineering experiments. During the past two decades, the *E. coli* K-12 host has been routinely used for production of a variety of substances on the industrial scale. *E. coli* K-12 strains do not have the sucrose fermenting capacity that other industrially useful microorganisms have. Therefore, the use of relatively inexpensive raw materials (e.g., molasses) in the fermentation process is not feasible. Sucrose fermenting *E. coli* K-12 strains could substantially reduce the cost of recombinant protein production. A two-plasmid system was successfully engineered to render *E. coli* K-12 sucrose-positive. In addition, high level recombinant target protein production was achievable with invertase containing strains grown on sucrose. Expression of recombinant PEP-C was compared in this strain grown on the three different media. PEP-C expression in cells grown on sucrose only was either comparable to or better than expression observed in cells grown either on glucose or in rich medium. These experiments demonstrated high level recombinant target protein production in an *E. coli* K-12 strain grown on sucrose as the sole carbon source in the growth medium.

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PREFACE

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1. INTRODUCTION

Escherichia coli K-12 strains were the most widely used microorganisms for carrying out genetic engineering experiments. As a result, during the past two decades the *E. coli* K-12 host has been routinely utilized for production of a variety of substances on the industrial scale. *E. coli* K-12 strains do not have the sucrose fermenting capacity that other industrially useful microorganisms have, therefore, the use of relatively inexpensive raw materials, e.g. molasses, in the fermentation process was not feasible. The use of sucrose fermenting *E. coli* K-12 strains could substantially reduce the cost of recombinant protein production.

Several attempts have been described to confer sucrose fermenting capability to *E. coli* K-12. Naturally occurring plasmids from a variety of bacterial sources were used to transfer sucrose utilization systems to *E. coli* K-12 either using genetic conjugation or by cloning these genes onto artificial plasmids and subsequent transformation or chromosomal integration via homologous recombination[1-7]. Two important aspects of this study make it very different from previous studies. (1) The genes of different sucrose utilization systems in earlier studies were usually obtained from bacterial sources other than *E. coli*. These genes were generally poorly characterized, and the results could not be correlated with a specific genetic background. In addition, stable maintenance of the sucrose positive phenotype often proved to be problematic. In our studies we used genes derived exclusively from the chromosomes of wild-type isolates of *E. coli*, therefore, greater compatibility and stability in *E. coli* K-12 was expected. Instead of using whole operons, we only included the essential genes of the utilization system, namely those coding for the transport protein (transporter) in the membrane and the sucrose-degrading enzyme (invertase) in the cytoplasm. (2) Model systems used in earlier studies focused on production of small molecular weight substances, e.g. amino acids or vitamins. To date, there has been no experimental data available on the benefits of a sucrose-fermenting *E. coli* K-12 strain in the production of recombinant proteins.

The two essential components of this model system are: 1) The recombinant protein target phosphoenolpyruvate-carboxylase (PEP-C). This gene has been previously cloned onto a high-copy number plasmid (pSP72; Promega Corp.) and successfully over-expressed to high levels (20-30% of total cytoplasmic protein) in

E. coli K-12 XLI-Blue. This protein can be purified by a single gel-filtration chromatographic step, and enzyme activity was readily measurable even from crude lysates. 2) Sucrose utilization (SUT) system. At least two genes were necessary and sufficient for sucrose uptake and metabolism, one encoding a transport protein (transporter) which facilitates the active uptake of sucrose through the inner (cytoplasmic) membrane; the other coding for a sucrose-splitting enzyme, invertase (INV), which was expressed in the cytoplasm. From previous studies it appears that transporter function was crucial for efficient growth on sucrose, therefore, we tested two transporters with different transport mechanisms, designated SUT1 and SUT2. The SUT1 gene was sequenced as part of a chromosomally encoded sucrose operon (*csc*) from a wild-type isolate of *E. coli*[8]. The product of this gene, a sucrose transport protein, was highly homologous to the lactose permease of *E. coli*, and catalyzes the active co-transport of H^+ and sucrose[8-10]. More recently, two genes from a clinical isolate of wild-type *E. coli*[7] encoding a novel sucrose-transport system (SUT2) have been sequenced (M. Sahin-Tóth, unpublished observations). Although the exact transport mechanism of this system has not been studied yet in detail, it belongs to a family of transporters which catalyze ATP-driven transport, therefore, it was reasonable to speculate that SUT2 was an ATP-driven sucrose transporter (M. Sahin-Tóth, unpublished observations). Since expression of different recombinant proteins may cause various metabolic changes in the cells, it was important to test transporters which have different mechanisms of transport and therefore may function differently under altered metabolic conditions. To ensure further metabolism of the imported sucrose, the gene coding for a cytoplasmic invertase (INV) from the *csc* operon[8] will be co-expressed with both transport systems.

The purpose of this paper is to report on the development a generally applicable model system of *E. coli* K-12 strain which expresses recombinant proteins to high levels and was able to utilize sucrose as the main carbon source in the growth-medium. Several sucrose fermenting strains were constructed, characterized and tested for production of a model recombinant protein, PEP-C. Finally, these findings were integrated in a genetic-metabolic model, with primary emphasis on defining the optimal genetic arrangement for high-level target protein production.

2. MATERIALS AND METHODS

2.1. Construction of *E. coli* strains harboring a sucrose utilization system.

Standard molecular biological methodology (e.g. PCR, restriction digestion, agarose gel-electrophoresis, plasmid purification, ligation, transformation) were used, as described in textbooks[11] and the manufacturer's instruction manuals. Plasmids carrying genes for SUT1, SUT2 and INV were used in the genetic manipulations. Since the primary focus in Phase I of the project was to define a genetic background best suited for high-level expression of PEP-C, genes for PEP-C and components of the sucrose-utilization systems were introduced into *E. coli* K-12 XL1-Blue using two different arrangements.

2.1.1. One-plasmid system.

Plasmids carrying the sucrose utilization system and the target gene were constructed. The vector backbone was a pUC-derived high-copy number plasmid (pSP73, Promega Corp.), and the design will allow for easy replacement of the target gene with other genes. Expression of PEP-C was controlled by its own constitutive promoter, while the sucrose transporters (SUT1 or SUT2), and the invertase gene were placed under the control of the lac promoter and their expression separately induced by lactose or isopropyl 1-thio-β-D-galactopyranoside (IPTG). The invertase gene (INV) was amplified by PCR from a plasmid previously described[8-10] and cloned into pSP73 using *Xho* I and *Hpa* I restriction sites (pSP73/INV). The forward oligonucleotide primer used for PCR amplification included the ribosome binding site (Shine-Dalgarno sequence) necessary for efficient translation of the INV gene, and the reverse primer incorporated a new *Pst* I site at the 3′ end, to facilitate further mobilization of the gene. The whole SUT1 gene with the lac promoter/operator region was mobilized by *EcoR* I and *Xho* I digestion from a plasmid described[9-10], and cloned into pSP73/INV (pSP73/SUT1-INV). The two genes of SUT2 were digested out (*EcoR* I, *Xho* 1) from a previously constructed plasmid (M. Sahin-Tóth, unpublished observations), carrying the SUT2 genes under the control of the lac promoter. The *EcoR* I - *Xho* I SUT2 fragment was ligated into pSP73/INV digested with the same enzymes, resulting in pSP73/SUT2-INV. The PEP-C gene was cut out from pSP72/PEP-C (previously constructed at our company), and cloned into pSP73/SUT1-INV and pSP73/SUT2-INV using *EcoR* I and *Nde* I restriction sites (pSP73/SUT1-INV/PEPC and pSP73/SUT2-INV/PEPC). Note that orientation of the PEP-C gene was opposite to the sucrose utilization genes.

2.1.2. Two-plasmid system.

Alternatively, the target gene (PEP-C) and the sucrose utilization system was expressed from two different plasmids. PEP-C had been previously cloned in the pUC derived plasmid pSP72 (Promega Corp.). SUT1 and SUT2 (under the control of the lac promoter) with the invertase gene was cloned in a vector which carried the origin of replication from plasmid p15A enabling it to co-exist in cells with pUC-derived plasmids. The clear advantage of a two-plasmid system was that no extra sub-cloning work was necessary when the sucrose utilization system was used with new target genes. On the other hand, plasmids with a pUC-derived origin of replication were present in much higher copy number than p15A-derived vectors. The entire sucrose utilization system was digested from pSP73/SUT1-INV and pSP73/SUT2-INV using *Bgl* 11 and *Pst* I restriction enzymes. The vector backbone carrying a p15A-derived origin of replication came from plasmid pGP1-2[12]. This plasmid was digested with *BamH* I and *Pst* 1, and ligated with the DNA fragments of the sucrose utilization genes. Note that *BamH* I and *Bgl* II generate compatible cohesive ends. The resulting plasmid carried a kanamycin resistance selection marker (pKAN/SUT1-INV and pKAN/SUT2-INV). In the two-plasmid system pKAN/SUT1-LNV and pKAN/SUT2-INV was used to co-transform *E. coli* K-12 XL1-Blue harboring plasmid pSP72/PEP-C, which was previously constructed at our company.

2.2. Characterization of Sucrose Utilization Capabilities.

In this experimental step, the "sucrose-positive phenotype" of each engineered strain was characterized. The goal was to better understand the effect of the different genotypes (i.e. different plasmid arrangements) on the strains' sucrose metabolism.

2.2.1 Comparison of growth in minimal medium with glucose or sucrose as sole carbon source.

The simplest approach to investigate whether or not the sucrose negative *E. coli* K-12 XLI-Blue was successfully transformed into a sucrose positive bacterium, was to test its ability to grow on media containing sucrose as the only carbon source. For rapid qualitative evaluation, cells were spread on agar-plates containing M9 minimal medium and 1% sucrose or 1% glucose as carbon source. Plates were grown at 37°C, and size of individual colonies compared by visual inspection after 12 and 24 hour growth period. Quantitative measurement of growth was carried out in liquid M9 medium with 1% sucrose or 1% glucose as carbon source (Table 1). Cultures were inoculated from fresh,

fully grown pre-cultures at a dilution of 1:100, and grown in a shaking incubator (250 rpm) and changes in optical density (OD) at 600 nm as a function of time were followed.

Table 1. M9 minimal medium constituents/liter

200 ml M9 salt solution (5x)
1 ml Trace-metal solution (1000x)
1 ml $MgSO_4$ (1M)
1 ml Thiamine (1%)
1 ml ampicillin (100 mg/ml)
5 ml kanamycin (10 mg/ml) - only for the two-plasmid system
0.5 ml 1-thio-β-D-galactopyranoside (1M)

Trace-metal solution (1000x/l)	M9 salt solution (5x/liter)
480 mg $FeCl_3 \cdot 6H_2O$	64 g $Na_2HPO_4 \cdot 7H_2O$
280 mg $MnCl_2 \cdot 4H_2O$	15 g KH_2PO_4
270 mg $CaCl_2$	2.5 g NaCl
2 g $ZnCl_2$	5.0 g NH_4Cl
290 mg H_3BO_3	
130 Mg $CoSO_4$	

2.2.2. Measurement of sucrose uptake (transport).

For preliminary qualitative assessment of sucrose transporter activity, *E. coli* K-12 XLI-Blue was transformed with given plasmids and the cells were plated on MacConkey agar indicator plates containing 30 mM sucrose, 100 μg/ml ampicillin, and 1 mM IPTG. For the two-plasmid system 50 μg/ml kanamycin was also included. Cells expressing functional sucrose transporters import sucrose, allowing metabolism of the monosaccharides released by invertase action. The consequent acidification resulted in the appearance of red colonies on the indicator plate. Color formation was proportional with sucrose uptake and metabolism. As a more quantitative measure of function, uptake of [^{14}C(U)] sucrose was followed in each strain. *E. coli* K-12 XLI-Blue cells harboring given plasmids were grown in Luria-Bertini (LB) broth containing 100 μg/ml ampicillin and 0.5 mM IPTG at 37°C overnight (12-14 hours). For the two-plasmid system 50 μg/ml kanamycin was also included. Cells were harvested by centrifugation, washed with 100 mM KPi (pH 7.5)/10 mM $MgCl_2$ and re-suspended in the same buffer to a final concentration of 10 OD_{600mm} per ml (~ 1 mg total protein/ml). Aliquots of cells (50 μl) were assayed at room temperature by rapid filtration. Transport was initiated by the addition of [^{14}C(U)] sucrose [5 mCi/mmol; 0.4 mM final sucrose concentration]. Reactions were quenched at given times by addition of 3.0 ml of 100 mM KPi (pH 5.5)/100 mM LiCl and rapidly filtered through Whatman GF/F filters. Filters were dried and radioactivity was counted in a liquid scintillation counter.

2.2.3. Measurement of invertase activity.

Function of the sucrose-splitting enzyme, invertase, was measured from fresh sonicated lysates of bacterial cells by following the appearance of reducing sugars (i.e. glucose). Cells were grown, harvested and washed as described above, and resuspended in 100 mM KPi pH 7.5/1 mM dithiothreitol. Cells were lysed by a brief ultrasound treatment (Sonic Dismembrator 550, Fisher Scientific, microprobe; setting 5, 3 times 15 seconds with 30 second cooling intervals, on ice). The sonicated suspension was centrifuged, and 0.5 ml of the supernatant incubated with 0.5 ml of 1M sucrose for 30 min at 37°C. The reaction was stopped by placing the samples in a boiling water bath for 5 minutes. The liberated glucose was then assayed by the hexokinase--glucose-6-phosphate dehydrogenase coupled enzymatic method using a commercial Glucose Reagent (Medical Analysis Systems, Inc.) according to the manufacturer's instructions.

2.3. Characterization of PEP-C Expression in the Sucrose-fermenting Strains

Complex interactions at the genetic and metabolic level influence the final output of target protein production. Metabolic factors affect gene-expression, which, in turn, may modify or offset the metabolic effects. In an attempt to isolate these effects and study them separately, we followed the expression of PEP-C under different genetic (SUT1 or SUT2, one-plasmid or two-plasmid system) and metabolic (growth on rich medium, and on minimal media with glucose or sucrose) conditions. As pointed out earlier, sucrose transport function may vary in different environments, resulting in altered growth rates and protein production. Similarly, depending on the extent of selective pressure(s) during growth, strains with the one-plasmid or the two-plasmid systems may exhibit drastically different target protein yields. All fermentation experiments were carried out in shaking cultures, at 37°C, in an Innova 4500 (New Brunswick Scientific) shaking incubator, using a setting of 250 rpm. Cultures were inoculated form fresh, fully grown pre-cultures at a dilution of 1:100, and grown in LB broth or M9 minimal medium with 1% glucose or sucrose as carbon source (see 3.21 above). One-milliliter samples were withdrawn after 3, 6, 12 and 24 hour growth, and analyzed for PEP-C expression. Bacterial cells were pelleted in an Eppendorf microcentrifuge (14k rpm; 2 min), and resuspended in 0.5 ml KPi (50 mM pH 7.5). The cell suspension was sonicated 3 times for 15 seconds (with 30 second cooling intervals) on ice, and cellular debris spun out in the microcentrifuge. The supernatant was collected, and PEP-C enzyme activity was measured using a microplate assay. In

addition, 10 µl samples were electrophoresed on 7.5% SDS-PAGE, and proteins were stained with Coomassie Brilliant Blue R 250. The amount of PEP-C visualized on gels was quantitated using a scanning densitometer (Molecular Dynamics). PEP-C enzyme activity was measured by a microplate assay. The assay was based on a coupled enzymatic reaction: phosphoenolpyruvate was carboxylated to oxaloacetate by PEP-C, and oxaloacetate was reduced to L-malate by malate dehydrogenase at the expense of NADH. Enzyme solution (100µl) was mixed with 100 µl of assay mixture, and the kinetics of NADH consumption was followed by measuring the decrease in absorbance at 340 nm. Contents of the assay mixture (final concentrations given): 100 mM Tris-Acetate pH 8.0, 1.17 M dioxane, 10 mM $KHCO_3$, 10 mM Mg-Acetate, 2 mM K-phosphoenolpyruvate, 1 mM NADH, 0.2 mM dithiothreitol, 1.5 IU malate dehydrogenase.

2.4. Characterization of PEP-C Expression in Molasses-containing Medium.

On the basis of the results from experiments described above, a best-performing strain (or possibly several strains) were selected, and used to optimize PEP-C production from molasses fermentation. Fermentation experiments and analysis of PEP-C production were carried out exactly as described above. For fermentation medium, M9 minimal medium containing 1%, 2%, 4% or 8% high-test molasses was used; growth and PEP-C production on different molasses concentrations were compared.

2.5. Evaluation of Experimental Data.

The final aim of this project was to outline a preliminary genetic-metabolic model for optimal expression of PEP-C in a sucrose-fermenting *E. coli* K-12 strain. In simplistic terms, our goal was to find the best PEP-C producing strain. In a broader sense, however, we would like not only to identify specific conditions required for high-level protein expression, but also to describe generally applicable rules which can help us in future design of recombinant protein producing strains. As pointed out earlier, even at the genetic level interactions modulating the expression of the target protein can be very complex. For example, in the one-plasmid-system, the increased plasmid size with multiple genes on the same plasmid may affect plasmid stability and copy number. Similarly, in the two-plasmid-system co-existence of two different plasmids within the same cell may influence their stability and copy-numbers. In both cases, the resulting negative or positive gene-dosage effect directly alters PEP-C expression, and by modifying expression of the sucrose-utilization system, it may also indirectly affect expression of the target protein. Given the complexity of the

possible interactions, our primary focus in Phase I was to define the best-functioning genetic arrangement: SUT1 vs. SUT2, one-plasmid system vs. two-plasmid system. Once this has been attained, optimization of growth on molasses and investigation of the effect of metabolic factors may begin. While an initial characterization of PEP-C production from molasses fermentation was planned for Phase 1 (see above), a more detailed elucidation of the role of metabolic factors and growth conditions was the objective of Phase 2.

3. RESULTS AND DISCUSSION

3.1. Construction of *E. coli* strains harboring a sucrose utilization system.

The primary focus in Phase I of the project was to define a genetic background best suited for high-level expression of PEP-C. In principle, at least two genes should be necessary and sufficient for sucrose uptake and metabolism, one encoding a transport protein which facilitates the uptake of sucrose through the inner (cytoplasmic) membrane; the other coding for INV, which was expressed in the cytoplasm. Despite continuous efforts we were unable to create a stable high copy-number plasmid (using the pUC-derived pSP73 vector) carrying both the SUT and PEP-C, as originally proposed ("one-plasmid system"). It appears that such a plasmid construct was toxic to the host cell. Therefore, we have concluded that such a genetic arrangement (i.e. the "one-plasmid system") was not feasible. Even though the final plasmid construct was not achieved, two interesting plasmids were constructed during these experiments which were later further characterized: pSP73/INV and pSP73/SUT1-INV. The pSP73/SUT1-INV plasmid contains a sucrose transporter and an invertase gene cloned under the control of the *lac*Z promoter/operator in a polycistronic fashion. The pSP73/INV plasmid was the precursor plasmid to pSP73/SUT1-INV, containing only the INV without any engineered promoter. Interestingly, invertase expression was observed from this plasmid, too, probably driven by other promoters or promoter-like structures on the plasmid (e.g. the promoter for the ampicillin resistance gene or the T7 promoter).

In view of the apparent problems with the one-plasmid system we focused our efforts on the construction of a viable two-plasmid system. In this system PEP-C was kept on a high copy-number plasmid (pSP72/PEP-C), and the sucrose utilization genes were separately introduced on a low copy-number vector. To minimize potential toxicity problems, we redesigned the two-plasmid system, and instead of using the lac promoter (as originally proposed), we cloned the invertase and the transporter

genes with their relatively weak constitutive promoters onto a pACYCI77 backbone. This new plasmid (pINV/SUT2/KAN) carries a kanamycin resistance gene, and an origin of replication compatible with that of pSP72/PEP-C. The pINV/SUT2/KAN plasmid was stable when introduced alone or along with pSP72/PEP-C into SM138 and no toxicity was observed. In addition, pINV (the invertase gene with its own promoter in a pBR322 vector) and pINV/KAN (invertase gene with its own promoter in a pACYC177 vector) were also constructed and characterized to some extent.

3.2. Characterization of sucrose-utilization capabilities of the engineered strains.

Selection of fast-growing *E. coli* K-12 strains. Most of our efforts were focused on experiments where characteristics of growth on rich medium and minimal medium containing glucose or sucrose as sole carbon source were compared. In our initial growth experiments we encountered problems associated with sluggish growth of certain *E. coli* strains on minimal medium. To overcome this problem, we tested a number of K-12 strains grown on M9 minimal medium supplemented with different combinations of magnesium, calcium, thiamine, and low concentrations of the amino acids proline and leucine (Table 2).

Table 2. M9 minimal medium supplements.

Experiment	1	2	3	4	5	6
Trace mix	X		X	X	X	X
Thiamine	X	X	X	X	X	X
$MgSO_4$				X	X	
$MgCl_2$	X	X	X			X
$CaCl_2$					X	X
Proline	X	X		X	X	X
Leucine	X	X		X	X	X

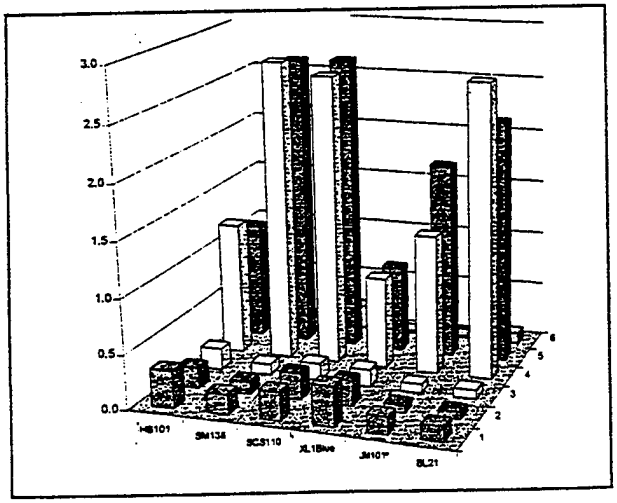

Figure 1. Selection of *E. coli* strains.

As shown in the figure, good growth was obtained with strains SM138 (a strain used in our laboratory for production of recombinant proteins under the control of the alkaline phosphatase promoter), and the commercial strains SCS110 and BL21. It was also apparent from this experiment that the use of

magnesium chloride instead of magnesium sulfate was inhibitory to growth (The vertical axis indicates overnight culture densities measured as OD at 600 nm). We selected strain SM138 for further characterization, and studied its magnesium (Mg), thiamine (B1), calcium (Ca) and trace metal (Tr) requirements. The results demonstrate that the presence of trace metals and magnesium sulfate was sufficient for high level growth in M9 minimal medium.

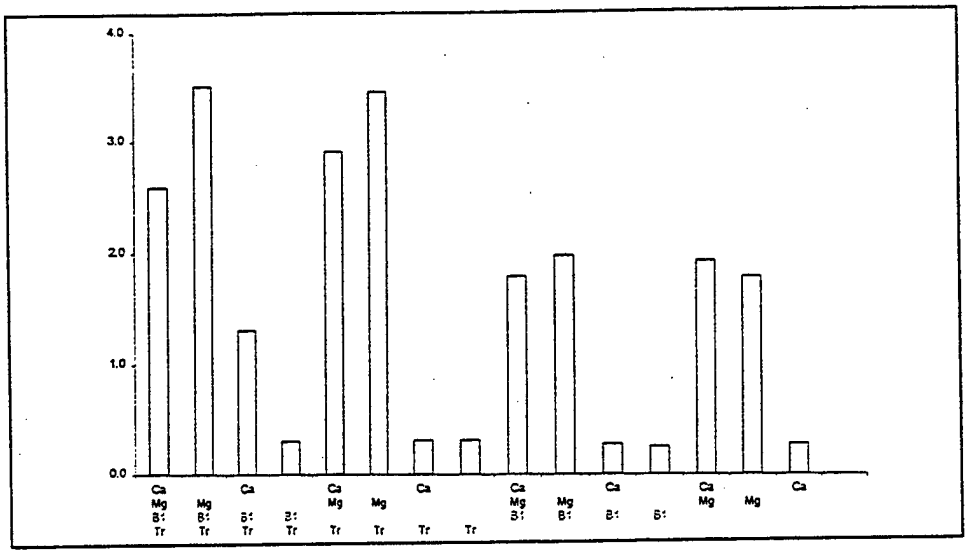

Figure 2. Metal requirements for strain SM138.

3.2.1. Plasmid stability in SM138.

Strain SM138 was transformed with different plasmids (pSP73/INV, pSP73/SUT1/INV, pINV/SUT2/KAN, pINV, and pSP72/PEP-C co-transformed with pINV/SUT2/KAN) and after overnight growth in rich LB medium plasmid DNA was prepared from each transformant and the plasmids were analyzed by agarose gel-electrophoresis. Ethidium-bromide staining of the gels shows that each plasmid was preserved in the transformed strains. Importantly, both plasmids were visualized in the DNA preparation from the co-transformed strain, carrying pSP72/PEP-C and pINV/SUT2/KAN.

3.2.2. Growth characteristics of SM138 carrying different plasmids.

SM138 harboring plasmids described above were grown on rich medium or minimal medium containing glucose or sucrose as carbon source. Growth was followed by measuring optical density of the cultures as a function of time. As expected, fast growth was observed in LB, with no significant difference between strains with different plasmids. Similar results were obtained with M9-glucose media. Unexpectedly, all strains grew to high levels in minimal medium containing only sucrose as carbon source. It was important to point out that plasmids containing

transporter and invertase genes (pSP73/SUT1/INV and pINV/SUT2/KAN) seem to grow at faster initial rate. Surprisingly, however, the presence of a transporter gene was not a requirement for growth on sucrose, and strains harboring only the invertase reach similarly high levels of growth (Figure 3).

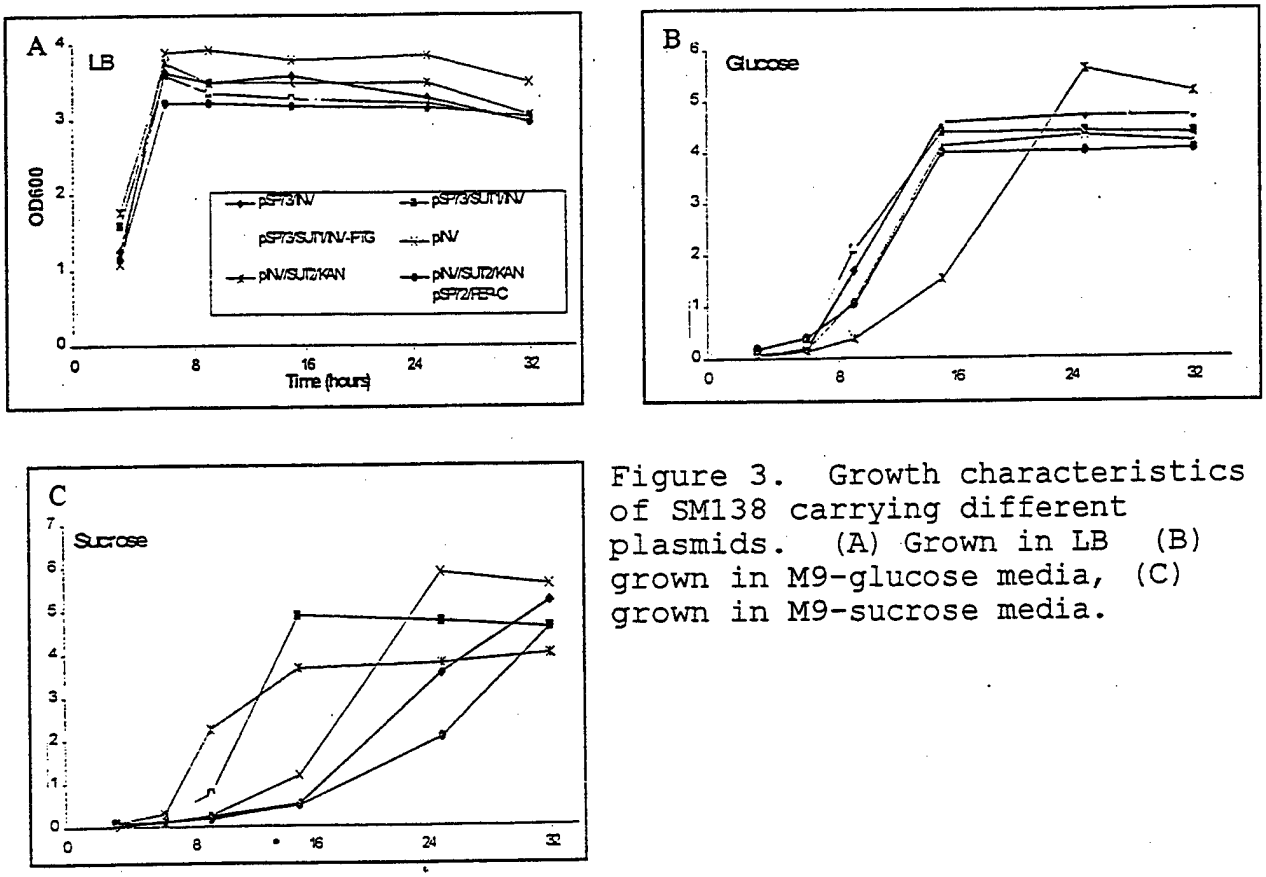

Figure 3. Growth characteristics of SM138 carrying different plasmids. (A) Grown in LB (B) grown in M9-glucose media, (C) grown in M9-sucrose media.

3.2.3. Measurement of invertase activity.

The presence of invertase activity was directly demonstrated in SM138 strains harboring plasmids pINV/SUT2/KAN and pINV/SUT2/KAN + pSP72/PEP-C. Invertase was assayed by following conversion of sucrose to glucose using a commercial glucose assay kit. As shown on Figure 4, total enzyme activity increases during exponential growth and stays constant as saturation was reached. Significant decline was observed between 24 and 50 hours of growth, even though culture densities exhibit very little decrease over this time period.

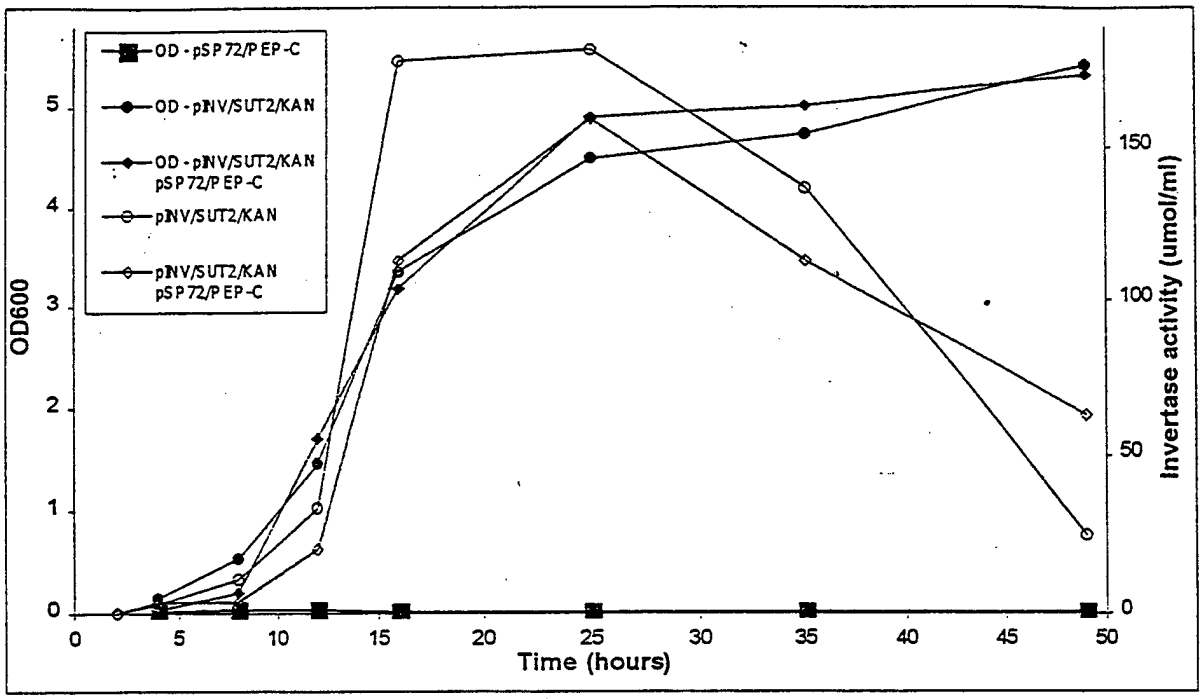

Figure 4. Measurement of Invertase Activity.

3.2.4. Sucrose uptake studies.

To demonstrate directly that these strains take up sucrose, transport experiments using radiolabeled sucrose were carried out. SM138 containing different plasmids was exposed to 0.4 mM [^{14}C]-labeled sucrose for 30 min. After filtration, radioactivity taken up inside the cells was counted Figure 5.

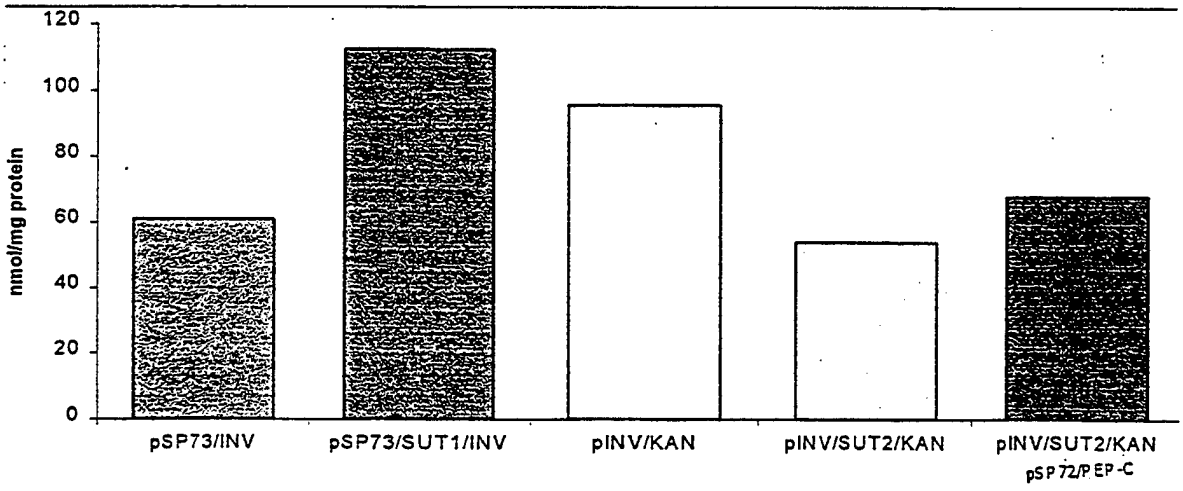

Figure 5. Sucrose Uptake in SM138 Plasmids.

As shown on Figure 5, each strain took up significant amounts of radioactivity. Again, strains with recombinant transporters seem to do better, but the invertase gene alone was sufficient to induce uptake of radioactivity from [^{14}C]-sucrose. Importantly, these results were in very good agreement with those of the growth experiments. There were at least two explanations for this phenomenon: (1) *E. coli* contains an intrinsic sucrose transporter, possibly another sugar transporter with some affinity towards sucrose at high enough concentrations; or (2) the invertase gene somehow "leaks" into the periplasmic space and degrades sucrose to glucose and fructose, and subsequently the monosaccharides were taken up and metabolized. However, transport experiments failed to show any sucrose accumulation by SM138 alone, therefore the possibility of a so far undiscovered sucrose transporter was unlikely. To gain better understanding of the phenomenon, we compared time-courses of uptake of sucrose in the presence and absence of 10 mM glucose (Figure 6).

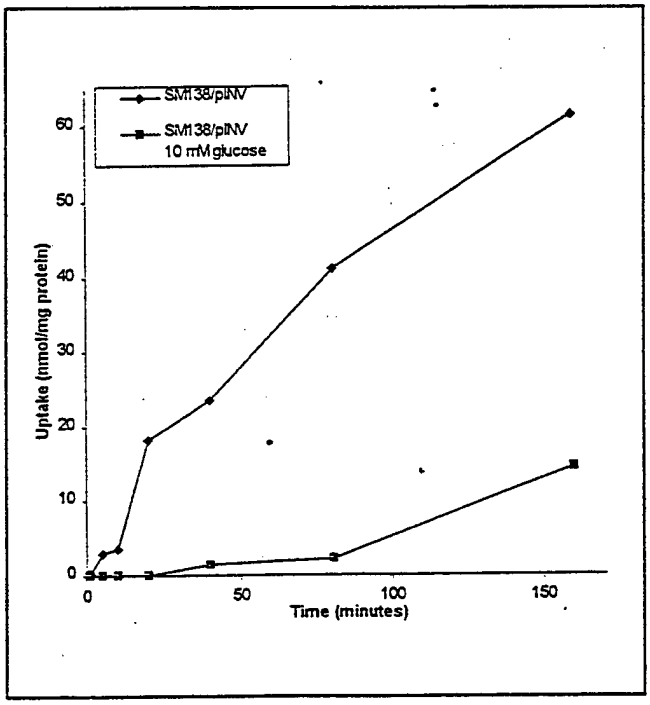

Figure 6. Time courses of ^{14}C-Uptake in Presence or Absence of Glucose.

Figure 6 demonstrates that radioactivity was accumulated as a function of time in a linear fashion, and glucose almost completely suppresses uptake. This observation strongly suggests that invertase was somehow exported from the cells and the outside invertase activity was responsible for the liberation of glucose (and fructose) from sucrose. In turn, the monosaccharides were metabolized. In a simple experiment we modeled the effect of extracellular invertase on apparent sucrose uptake. SM138 cells with no plasmid were incubated with [^{14}C]-sucrose for 20 min in the presence of 2 µL cytoplasmic fractions prepared from SM138/pINV cells. Indeed, addition of the crude enzyme significantly increased the uptake of radioactivity (not shown) indicating that the invertase present liberates glucose, which was freely available for transport.

3.2.5. Periplasmic export of invertase protein.

Arguably the most intriguing observation from these studies was that *E. coli* K12 cells harboring only a recombinant invertase gene were able to grow on relatively high concentrations of sucrose. While the mechanism of invertase export remains to be elucidated, it was important to note that similar observations were made when a sucrose utilization system was cloned from *Vibrio alginolyticus* into *E. coli* K-12. The parent strain exhibited no invertase export. For reasons that were not apparent, a fraction of the cytoplasmic invertase was exported to the periplasm by *E. coli* K-12 strains. In any event, the biotechnological implications of this observation were important: introduction of a sole gene was sufficient to render *E. coli* K-12 sucrose-positive. In addition, as demonstrated in the next paragraph, high level recombinant target protein production was achievable with invertase containing strains grown on sucrose.

3.3. PEP-C Expression in strain SM138 harboring pINV/SUT2/KAN and pSP72/PEP-C.

Expression of recombinant PEP-C was compared in this strain grown on the three different media. Enzyme activity was measured and the expressed protein was also visualized with the help of SDS-PAGE and Coomassie Blue staining. In two separate experiments, PEP-C expression in cells grown on sucrose only was comparable to or better than expression observed in cells grown on glucose or in rich medium. Figure 7 shows the results of the second experiments. [The arrow indicates the migration position of PEP-C. The last lane contains commercial PEP-C preparation with BSA present as stabilizer.]

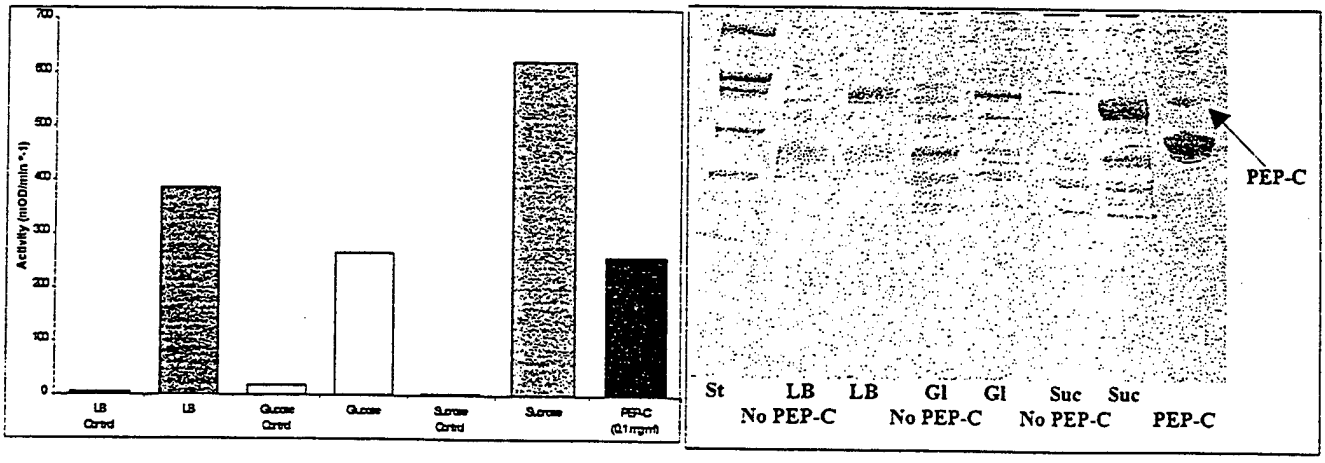

Figure 7. PEP-C Expression in Strain SM138.

These experiments prove the feasibility of the project described in our proposal: we successfully demonstrated high level recombinant target protein production in an *E. coli* K-12 strain grown on sucrose as the sole carbon source in the growth medium. Further experiments aimed at identifying optimal growth conditions for PEP-C production lead to the following conclusions: 1) Initial growth of the SM138 strain with the two plasmids was usually delayed compared to the parent strain or strains carrying only one plasmid. The reasons for this were at least two-fold: the presence of two plasmids was slightly toxic to these cells and depresses growth even in rich media; and periplasmic accumulation of the exported invertase and invertase action to generate growth-supporting concentrations of glucose takes more time. The extended lag-phase of growth can be shortened by increasing the amount of inoculate, dilutions 1:100 or smaller give satisfactory results. 2) PEP-C expression was variable when the co-transformed strain was inoculated from plates several weeks old or from "re-streaked" plates. This phenomenon was commonly observed with different protein expression systems of low-level toxicity, and can be overcome by using fresh transformants for protein production.

3.4. Growth of sucrose-fermenting SM138 strain on molasses.

The ultimate purpose of engineering sucrose-fermenting strains was to be able to utilize inexpensive growth-media, like molasses, during large-scale fermentation. Molasses contains mostly sucrose and sucrose-fermenting strains should be able to grow to high densities on media with molasses as carbon source. During the final period of Phase I, initial experiments were carried out to obtain some level of understanding on the feasibility of molasses-supported growth of the engineered strains. Using molasses obtained from a local grocery store, the following observations were made: 1) there was no significant difference between growth kinetics and final culture densities of sucrose-fermenting and non-sucrose-fermenting strains when grown on minimal media containing molasses and 2) steady-state culture density appeared to be determined by the glucose content of the molasses.

This Phase I project achieved its important goals. A sucrose-fermenting *Escherichia coli* K-12 strain was successfully engineered, and it was demonstrated that high-level recombinant protein production was feasible using growth media with sucrose as the sole carbon source. These results set the stage for Phase II of the project, which will be aimed at identification and optimization of fermentation conditions for low-cost overproduction of recombinant proteins using the engineered sucrose-fermenting *E coli* K-12 strains.

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